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Chitinase Inhibition Promotes Atherosclerosis in Hyperlipidemic Mice

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Chitinase 1 (CHIT1) is secreted by activated macrophages. Chitinase activity is raised in atherosclerotic patient sera and is present in atherosclerotic plaque. However, the role of CHIT1 in atherosclerosis is unknown. Preliminary studies of atherosclerosis in cynomolgous monkeys revealed CHIT1 to be closely correlated with areas of macrophage infiltration. Thus, we investigated the effects of a chitinase inhibitor, allosamidin, on macrophage function *in vitro* and on atherosclerotic development *in vivo*. In RAW264.7 cells, allosamidin elevated monocyte chemoattractant protein 1 and tumor necrosis factor alpha expression, and increased activator protein 1 and nuclear factor-κB transcriptional activity. Although inducible nitric oxide synthase, IL-6, and IL-1β expression were increased, Arg1 expression was decreased by chitinase inhibition, suggesting that suppression of CHIT1 activity polarizes macrophages into a M1 phenotype. Allosamidin decreased scavenger receptor AI, CD36, ABCA1, and ABCG1 expression which led to suppression of cholesterol uptake and apolipoprotein AI-mediated cholesterol efflux in macrophages. These effects were confirmed with CHIT1 siRNA transfection and CHIT1 plasmid transfection experiments in primary macrophages. Apolipoprotein E-deficient hyperlipidemic mice treated for 6 weeks with constant administration of allosamidin and fed an atherogenic diet showed aggravated atherosclerotic lesion formation. These data suggest that CHIT1 exerts protective effects against atherosclerosis by suppressing inflammatory responses and polarizing macrophages toward an M2 phenotype, and promoting lipid uptake and cholesterol efflux in macrophages. *(Am J Pathol 2013, 183: 313–325; http://dx.doi.org/10.1016/j.ajpath.2013.04.003)*

Atherosclerosis is characterized by excessive lipid accumulation and chronic inflammation within the arterial wall.[1] When risk factors such as hyperlipidemia and hypertension are presented, vascular endothelial cells are stimulated to express adhesion molecules, which promote attachment of circulating leukocytes. Concomitantly, increased endothelial permeability allows deposition of cholesterol-containing lipoproteins in the intima. These lipoproteins can become biologically or chemically modified and stimulate surrounding cells to express chemokines such as monocyte chemoattractant protein 1 (MCP-1), which can induce further leukocyte migration into the vascular wall. Monocytes are one of the first leukocytes to transmigrate and differentiate into macrophages, which are the predominant immune cells observed in atherosclerotic lesions, especially in the early stages. Macrophages possess an unlimited propensity to take up accumulated modified lipoproteins such as oxidized low density lipoprotein via scavenger receptors (SR) and thereby get transformed into cholesterol-rich foam cells that make up the majority of the

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early fatty streaks. Macrophages activated by modified lipoproteins produce pro-inflammatory cytokines, including IL-1β and tumor necrosis factor α (TNFα), which can mediate inflammation in the arterial wall and cause subsequent infiltration of T lymphocytes that may further accelerate inflammation in the lesion. Activated macrophages also produce reactive oxygen species, matrix metalloproteinases that degrade extracellular matrix, and the procoagulant tissue factor, which induce subsequent plaque rupture and thrombogenesis.[2] Thus, macrophages play a pivotal role in the pathogenesis of lesion formation throughout all stages of atherosclerosis.

The enzyme chitotriosidase [chitinase 1 (CHIT1)] is a member of the glycosyl hydrolase family 18, which cleaves its substrate, chitin.[3] Chitin is a polymer of β-1, 4-N-acetyl-glucosamine, which is produced by many living organisms including insects, fungi, crustaceans, and other marine organisms. Interestingly, despite the absence of chitin in mammals, CHIT1 is abundantly produced by activated macrophages in various human diseases, including Gaucher disease and Niemann-Pick disease. It has also been reported that chitinase activity is elevated up to 55-fold in human atherosclerotic tissue.[4] Moreover, serum chitinase activity is elevated in patients with atherosclerosis and is associated with the severity of atherosclerotic lesions.[5] Similarly, when we performed a comprehensive gene expression analysis by DNA microarray assay, we found a tight correlation between CHIT1 mRNA expression and areas of macrophage infiltration in atherosclerotic lesions of cynomolgus monkeys. However, little is known about the physiological role of CHIT1 in the pathogenesis of atherosclerosis. In this study, we investigated the effects of a chitinase inhibitor, allosamidin,[6] on macrophage function and atherosclerotic lesion development to speculate how CHIT1 activity might function in atherosclerosis.

Materials and Methods

Cell Culture

The mouse macrophage cell line RAW264.7 (American Type Culture Collection, Rockville, MD) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (50 U/mL), streptomycin (50 μg/mL), and L-glutamine (2 mmol/L). Cells were kept at subconfluence in a humidified incubator at 37°C with 5% CO_2. RAW264.7 cells were treated with 10 μmol/L allosamidin to inhibit chitinase activity. Bone marrow–derived macrophages (BMDM) from C57BL/6 mice were generated by flushing the femurs of mice.[7] After centrifugation at 300 × *g*, the cells were resuspended in macrophage growth medium consisting of DMEM and 10% fetal bovine serum, and were supplemented with 20% (v/v) L929 cell conditioned medium as a source of colony-stimulating factor, penicillin (100 U/mL) and streptomycin (100 mg/mL). Fibroblasts were allowed to adhere for 4 hours at 37°C and then nonadherent cells were transferred to another flask. This step was repeated after overnight incubation at 37°C. On day 8, most of the adherent cells were macrophages (>98% MAC1-positive cells and 99% phagocytosis activity). Isolated BMDM were stimulated with acetylated low density lipoprotein (Ac-LDL, 50 μg/mL) (Life Technologies, Grand Island, NY); monocyte chemoattractant protein-1 (MCP-1, 100 ng/mL, R&D Systems, Minneapolis, MN); lipopolysaccharide (LPS, 100 ng/mL) (Sigma-Aldrich, St. Louis, MO); advanced glycation end product bovine serum albumin (AGE-BSA, 100 μg/mL, Calbiochem, La Jolla, CA); or interferon gamma (IFNγ) (100 units/mL, eBioscience, San Diego, CA). The cells were also treated with 20 μmol/L allosamidin or vehicle control ± 10 units/mL IFN-γ, and used for the CHIT1 plasmid transfection study. Thioglycollate-elicited peritoneal macrophages were obtained from male C57BL/6 mice by standard method. In brief, mice were injected intraperitoneally with 1 mL of 3% Brewer thioglycollate medium (Becton Dickinson, Franklin Lakes, NJ). Four days later, the peritoneal cells were harvested by lavage with cold PBS and plated on petri dishes. The cells were allowed to adhere for 4 hours and washed with PBS to remove nonadherent cells before being used for the siRNA transfection study.

Chitinase Activity Assay

Chitinase activity assay was performed using 4-methylumbelliferyl-N, N′, N″-triacetyl chitotrioside [4MU-$(GlcNAc)_3$] (Sigma-Aldrich, St. Louis, MO) as a substrate.[8] A solution of 25 μmol/L 4MU-$(GlcNAc)_3$ in N, N-dimethylformamide was diluted 1:500 with distilled water, and the resulting solution (40 μL) was added to 0.5, 2, or 4 μL mixture of the culture filtrate in each experiment along with 50 mmol/L citric acid (50 mmol/L Na_2HPO_4 buffer; pH 5.0; 100 μL). After incubating the mixture at 28.5°C for 60 minutes, the reaction was stopped by adding 1.0 M glycine (NaOH buffer; pH 10.5; 2.5 mL), and the liberated 4-methylumbelliferone was measured with a fluorescence spectrometer (excitation at 360 nm, emission at 450 nm). The value of fluorescence strength was used for calibration of relative chitinase activity or calculation of absolute chitinase activity (U/mL) by referring to standard 4-methylumbelliferone solution. [Unit definition: One unit will release 1.0 μmole of 4-methylumbelliferone from 4MU-$(GlcNAc)_3$ per minute].

RNA Extraction and Real-Time RT-qPCR

Total cellular RNA was extracted from cells using TRIzol (Invitrogen, San Diego, CA), according to the recommended procedure. Real-time quantitative RT-PCR (real-time RT-qPCR) was performed using the ABI Prism 7700 (Applied Biosystems, Foster City, CA) SYBR Green analysis. The sequence-specified PCR primers used in these experiments

Table 1 Sequence-Specific PCR Primers Used for Real-Time RT-qPCR

Gene name		Primers
GAPDH	Forward	5′-GGCAAATTCAACGGCACAGT-3′
	Reverse	5′-CGCTCCTGGAAGATGGTGAT-3′
CHIT1	Forward	5′-CGTCATCTTTGCTTTTGCTGG-3′
	Reverse	5′-TGGTAGAGAAGTTCGTCATTGTGC-3′
MCP-1	Forward	5′-TCACTGAAGCCAGCTCTCTCT-3′
	Reverse	5′-GTGGGGCGTTAACTGCAT-3′
TNFα	Forward	5′-CTGTAGCCCACGTCGTAG C-3′
	Reverse	5′-GGTTGTCTTTGAGATCCATGC-3′
CCR2	Forward	5′-AGCACATGTGGTGAATCCAA-3′
	Reverse	5′-TGCCATCATAAAGGAGCCA-3′
CD36	Forward	5′-CAAGCTCCTTGGCATGGTAGA-3′
	Reverse	5′-TGGATTTGCAAGCACAATATGAA-3′
SR-AI	Forward	5′-TTAAAGGTGATCGGGGACAAA-3′
	Reverse	5′-CAACCAGTCGAACTGTCTTAAG-3′
ABCA1	Forward	5′-CCCAGAGCAAAAAGCGACTC-3′
	Reverse	5′-GGTCATCATCACTTTGGTCCTTG-3′
ABCG1	Forward	5′-CAAGACCCTTTTGAAAGGGATCTC-3′
	Reverse	5′-GCCAGAATATTCATGAGTGTGGAC-3′
PPARγ	Forward	5′-GTCACGTTCTGACAGGACTGTGTGAC-3′
	Reverse	5′-TATCACTGGAGATCTCCGCCAACAGC-3′
LXRα	Forward	5′-AGGAGTGTCGACTTCGCAAA-3′
	Reverse	5′-CTCTTCTTGCCGCTTCAGTTT-3′
iNOS	Forward	5′-CAAGCTGAACTTGAGCGAGGA-3′
	Reverse	5′-TTTACTCAGTGCCAGAAGCTGGA-3′
IL-6	Forward	5′-CCACTTCACAAGTCGGAGGCTTA-3′
	Reverse	5′-GCAAGTGCATCATCGTTGTTCATAC-3′
IL-12p35	Forward	5′-TGTCTTAGCCAGTCCCGAAACC-3′
	Reverse	5′-TCTTCATGATCGATGTCTTCAGCAG-3′
IL-1β	Forward	5′-TCCAGGATGAGGACATGAGCAC-3′
	Reverse	5′-GAACGTCACACACCAGCAGGTTA-3′
Arg1	Forward	5′-AGCTCTGGGAATCTGCATGG-3′
	Reverse	5′-ATGTACACGATGTCTTTGGCAGATA-3′
FIZZ1	Forward	5′-TCCAGCTGATGGTCCCAGTG-3′
	Reverse	5′-GAGGCCCATCTGTTCATAGTCTTG-3′
IL-10	Forward	5′-GACCAGCTGGACAACATACTGCTAA-3′
	Reverse	5′-GATAAGGCTTGGCAACCCAAGTAA-3′

ABCA1, ATP-binding cassette transporter A1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *iNOS*, inducible nitric oxide synthase; *LXRα*, liver X receptor α; *PPARγ*, peroxisome proliferator-activated receptor γ; *SR-AI*, scavenger receptor AI.

are listed in Table 1. The gene products were quantified using SYBR Green assays (Applied Biosystems, Foster City, CA) and glyceraldehyde-3-phosphate dehydrogenase was used for normalization.

Electromobility Shift Assay

Cells were cultured on a 12-well plate overnight. For collection of nuclear protein, cells were rinsed with PBS, removed from the plate with a cell scraper, transferred to a microfuge tube, and centrifuged at 4000 rpm for 5 minutes. The cell pellets were resuspended in 400 μL of cold 1× Solution A buffer [10 mmol/L HEPES (pH 7.4), 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L dithiothreitol, 0.4 mmol/L phenylmethylsulfonyl fluoride]. After resuspension, samples were incubated for 20 minutes on ice and vortexed for 1 minute. Samples were centrifuged at 600 × *g* for 10 minutes, and supernatant was completely removed. Pellets were resuspended in 1 mL of Solution C buffer containing 20 mmol/L HEPES (pH 7.4), 0.42 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.5 mmol/L dithiothreitol, 0.4 mmol/L phenylmethylsulfonyl fluoride and were placed on ice for 20 minutes. Samples were spun at 13,000 rpm for 10 minutes, and supernatants were transferred to a fresh tube. Protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL)) per the manufacturer's directions. Nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) consensus oligomer (50 ng) (Santa Cruz Biotechnology, Santa Cruz, CA) were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (TAKARA BIO INC, Ohtsu, Japan). The sequence of each consensus oligomer is described as follows. Nuclear protein (5 μg) was reacted with 20,000 cpm of labeled oligomer in binding buffer containing 20 mmol/L HEPES, 60 mmol/L KCl, 4% Ficoll, 0.1 mg/mL bovine serum albumin (BSA), 2 mmol/L dithiothreitol, and 0.1 mg/mL poly(dI-dC) (Roche, Basel, Switzerland) on ice for 30 minutes and was analyzed on a 6% acrylamide gel (80:1 ratio of acrylamide to bis-acrylamide). Gels were dried and exposed to X-ray film (Amersham, Arlington Heights, IL). For cold competition, 100-fold excess of unlabeled oligomer was added. The sequence of each consensus oligomer was: NF-κB: 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and AP-1: 5′-CGCTTGATGACTCAGCCGGAA-3′.

Reporter Gene Assay

2×10^5 RAW264.7 cells were prepared in a 24-well plate. The cells were then transfected with 0.7 μg/well of AP-1 or NF-κB promoter−luciferase plasmid (Clontech) and 0.3 μg per well of β-galactosidase gene driven by SV40 promoter-enhancer sequence (Promega). The transfection was performed using SuperFect transfection reagent according to the manufacturer's instructions (Qiagen, Valencia, CA). After incubation for 24 hours, cells were treated with 10 μmol/L of allosamidin or control vehicle for 6 hours. The cells were washed twice with phosphate-buffered saline, lyzed in 200 μL lysis buffer (25 mmol/L Tris, pH 7.8, 2 mmol/L EDTA, 2 mmol/L DL-dithiothreitol, 10% glycerol, and 1% Triton X-100), and 100 μL of lysate was used for luciferase activity assay in a Lumat luminometer (LB 9501) (Berthold Technologies, Oak Ridge, TN). The assay was started by adding 100 μL of 470 mmol/L luciferin to cell lysate, and integrated peak luminescence was determined over a 55-second window after a 5-second delay. The β-galactosidase activity in the same sample was measured spectrophotometrically and used to normalize the luciferase activity.

Binding and Uptake of AcLDL

To determine the receptor-specific binding and uptake, fluorescence-labeled acetylated LDL (DiI-AcLDL) was used

as previously described.[9] Cells seeded in a 12-well plate or chamber slide were treated with allosamidin overnight followed by incubation with DiI-AcLDL at 10 μg/mL in medium for 2 hours at 37°C. The media containing DiI-AcLDL was removed from culture and the cells were washed twice with probe-free media. Cells from 12-well plate were analyzed using a FACScan flow cytometer (Becton Dickinson), and data were analyzed with the CellQuest Pro version 5.1 (Becton Dickinson). Cells on the chamber slides were observed under fluorescence microscope.

Cholesterol Efflux Assay

RAW264.7 cells were seeded in a 24-well plate for incubation overnight before being labeled with loading media [DMEM/glutamine/p/s/10% lipoprotein-deficient calf serum 1 μCi/μL (of [^{3}H] cholesterol)] with or without 10 μmol/L allosamidin for 36 hours. The cells were washed twice with PBS, after which DMEM/glutamine/0.2% fatty acid-free BSA ± 10 μmol/L allosamidin was added into each well and incubated for 1 to 2 hours. The medium was then aspirated. For apolipoprotein AI (apoAI) and high-density lipoprotein (HDL)-independent cholesterol efflux assay, efflux medium (DMEM/glutamine/0.2% fatty acid-free BSA, ± 10 μmol/L allosamidin) without apoAI and HDL was added to one set of wells. For apoAI or HDL-dependent cholesterol efflux assay, efflux medium, as previously mentioned, along with 20 μg/mL of apoAI or 50 μg/mL of HDL was added in another set of wells. After incubation for 2 hours, 100 μL of media was removed and transferred to 1.5 mL Eppendorf tube. Cell debris was spun down by centrifuging for 5 minutes. The supernatant was gently transferred to 5 mL counting fluid to measure radioactivity [as effluxed cholesterol (5-minute program)]. At the end of the experiment, 0.5 mL of 0.1 N NaOH was added to lyze the cells by incubating for 5 hours at room temperature, and 100 μL of cell lysate was transferred to 5 mL counting fluid to measure radioactivity. ApoAI or HDL-induced [^{3}H] cholesterol efflux was measured as the fraction of total radiolabeled cholesterol appearing in the medium in the presence of apoAI or HDL after subtraction of values for apoAI and HDL-free medium. ApoAI or HDL-mediated efflux was determined using the following formula: i) percentage of efflux = efflux/total labeled cholesterol; ii) apoAI or HDL specific efflux = percentage of efflux (with apoAI or HDL) − the percentage of efflux (without apoAI and HDL); and iii) percentage of increase in apoAI or HDL-mediated efflux = percentage (apoAI or HDL-specific efflux)/percentage of efflux (without apoAI and HDL).

siRNA Transfection

Thioglycollate-elicited peritoneal macrophages were transfected with Dharmacon siGENOME SMARTpool siRNA (Thermo Fisher Scientific, Fife, WA) against mouse CHIT1 or siGENOME nontargeting siRNA pool using lipofectamine siRNAMax (Invitrogen), according to the manufacturer's instructions (final RNA interference duplex concentration, 20 nmol/L). After 48 hours of transfection, the cells were harvested for mRNA extraction and real-time RT-qPCR was performed.

CHIT1 Gene Transfection

Total mRNA was extracted from the mouse tongue using TRIzol, and mouse CHIT1 cDNA was amplified by using real-time PCR using two primers specific for the mouse *Chit1* gene (the sense primer: 5′-ACAGAGCTGATA-TCCCCAGAGCCT-3′; the antisense primer: 5′-TGGA-GTTGGATGGGGTTCCAGG-3′). The cDNA fragment was cloned into the pTAC-2 TA cloning vector (BioDynamics, Tokyo, Japan) and was further subcloned into the eukaryotic expression vector pcDNA3.1 plasmid. Afterward, BMDM were transfected with control pcDNA3.1 or pcDNA3.1-CHIT1 plasmid using MicroPorator MP-100 (Digital Bio Technology Co., Ltd., Seoul, Korea) according to the manufacturer's instructions. In brief, the cells were resuspended in Neon resuspension buffer R (Invitrogen) containing pcDNA3.1 or pcDNA3.1-CHIT1 plasmid (6 million cells per DNA, 8 mg/100 μL) and were pulsed twice with a voltage of 800 V and a width of 50 msec using a 100 μL Neon tip (Invitrogen). The cells were then quickly transferred into two 6 cm cell culture plates containing DMEM supplemented with 10% fetal bovine serum. After 48 hours of transfection, the cells were stimulated with or without 10 units/mL IFN-γ for 6 hours and harvested for mRNA extraction, and real-time RT-qPCR was performed.

Animal Models

Ten week old male apolipoprotein E (*ApoE*)-knockout mice (Jackson Laboratory, Bar Harbor, ME) were fed a chow diet or a high fat diet containing 35% (w/w) fat and 1% cholesterol (KBT Oriental Co., Ltd., Tosu, Saga, Japan) with 1.9 mg/kg per day infusion of angiotensin II by osmotic mini pump model 2004 (ALZET, Cupertino, CA) for 4 weeks, and the descending aortas were harvested. Total mRNA was extracted from the aortas and CHIT1 mRNA expression was evaluated by real-time RT-qPCR.

For the atherosclerotic mouse model of CHIT1 inhibition, spontaneous hyperlipidemic C57BL/6.KOR/Stm Slc-*Apoe*^{shl} mice, deficient in ApoE expression, were obtained from Japan SLC Inc. (Shizuoka, Japan).[10] Mice (12-weeks-old) were implanted subcutaneously with osmotic mini pump model 2006 (ALZET) containing 10 mg/kg per day allosamidin in 0.1 N acetic acid or 0.1 N acetic acid as a control vehicle, and given the Western diet containing 40% w/w fat and 0.15% cholesterol (KBT Oriental Co, Ltd.) for 6 weeks. Blood was taken every 3 weeks for lipid analysis and chitinase activity measurement in circulation. Systolic blood pressure and heart rate were evaluated by the tail-cuff method. The mice were maintained in the animal facilities at Kyushu University. The

study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments at Kyushu University Graduate School of Medical Sciences.

Analysis of Atherosclerosis and Other Measurements in Plasma

Mice were euthanized after 6 weeks of Western diet and perfused via the left ventricle with 10 mL PBS ($n = 6$ per group). The heart and aortic tree were dissected. Aortic roots were fixed in 4% paraformaldehyde and 5% sucrose, and embedded in OCT compound. The aortas were fixed in 10% formaldehyde overnight. Atherosclerotic lesions of the aortic sinus, as well as the entire length of the aorta, were analyzed as previously described.[11] Frozen sections (6 μg) of the aortic sinus were prepared, and the sections were used for detecting lipid (0.5% Oil Red O; Sigma-Aldrich), macrophages (mac-3, 1:1000; Pharmingen, San Diego, CA), and T cells (CD4, 1:100; Pharmingen) in the lesion. The atherosclerotic lesion stained with Oil Red O was quantitated using a computer-assisted imaging system (ImageJ version 1.41; NIH, Bethesda, MD). Lesion cell contents were determined by measuring the absolute positive area (macrophages) in the aortic sinus, or by counting the cells (T cells) in the intimal lesions. The plasma samples collected from the dorsal pedal vein of the mice were used to measure plasma total cholesterol and triglyceride levels to see whether allosamidin had an effect on these circulating lipids, as well as for plasma chitinase activity.

Statistical Analysis

Results are given as means ± SEM. Statistical analysis was performed by unpaired Student's *t*-test and one-way analysis of variance in comparing the differences between two groups and among multiple groups, respectively. A P value < 0.05 was considered significant.

Results

CHIT1 Expression Correlates with Atherosclerotic Lesion Area in Cynomolgous Monkeys

We previously reported that statin or anti−MCP-1 therapy resulted in regression and stabilization of atherosclerotic lesions, independent of cholesterol levels in nonhuman primates.[12] In this experiment, DNA microarray assay was performed with mRNA from atherosclerotic lesions of descending aorta to explore atherosclerosis-related genes, and CHIT1 was found to be one of 11 candidate genes that were closely correlated with treatment with pravastatin and 7ND transfection. Real-time PCR results confirmed that CHIT1 mRNA expression was reduced in pravastatin or 7ND-treated group (Supplemental Figure S1A). Furthermore, CHIT1 mRNA expression was also highly correlated with areas of macrophage infiltration in the atherosclerotic lesions of abdominal aortas (Supplemental Figure S1B).

CHIT1 Expression Is Upregulated by Pro-Atherosclerotic Stimuli in BMDM

IFN-γ, TNFα, and LPS are reported to increase CHIT1 mRNA expression.[13] To examine whether other pro-atherosclerotic stimuli upregulate CHIT1 mRNA expression, BMDM were treated with Ac-LDL, AGE-BSA, MCP-1, LPS, or IFN-γ. Although stimulation with Ac-LDL, AGE-BSA, LPS, and IFN-γ increased CHIT1 mRNA expression, MCP-1 had no effect on CHIT1 mRNA expression (Figure 1A).

Chitinase Inhibition Increases Pro-Inflammatory Cytokine Expression in Macrophages and Polarizes Macrophages into a M1 Phenotype

We used allosamidin to inhibit CHIT1 *in vitro* and *in vivo*. Chitinase activity was suppressed by allosamidin in a dose-dependent manner, with 10 μmol/L of allosamidin suppressing chitinase activity by more than 90% *in vitro* (Supplemental Figure S2). Because inflammation is one of the most important factors in the pathogenesis of atherosclerosis, we investigated the effects of allosamidin on pro-inflammatory cytokine expression in macrophages. Allosamidin treatment increased MCP-1 and TNFα mRNA expression in RAW264.7 macrophages, but did not change CCR2 expression (Figure 1B). To determine whether these changes were mediated via activation of transcriptional factors, we performed electromobility shift assay and reporter gene assay for AP-1 and NF-κB, and found that transcriptional activities of both AP-1 and NF-κB were modestly increased by allosamidin treatment (Figure 2,A and B). We also investigated whether allosamidin would influence macrophage polarization. Expression of M1 markers, inducible nitric oxide synthase, IL-6, and IL-1β, were increased, whereas expression of a M2 marker, Arg1, was decreased by chitinase inhibition (Figure 1B). Although not statistically significant, allosamidin treatment tended to decrease another M2 marker, Fizz1 expression, but raised IL-10 expression (Figure 1B). Collectively, these trends indicate that CHIT1 in its uninhibited state would favor M2 polarization.

Allosamidin Decreases Cholesterol Uptake and Efflux in Macrophages

Because lipid-laden macrophages also play an important role in the pathogenesis of atherosclerosis, we investigated the effects of allosamidin on cholesterol metabolism in macrophages. Allosamidin treatment decreased ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1), scavenger receptor AI (SR-AI), and CD36 mRNA expression in RAW264.7 cells (Figure 3A). Peroxisome proliferator-activated receptor γ

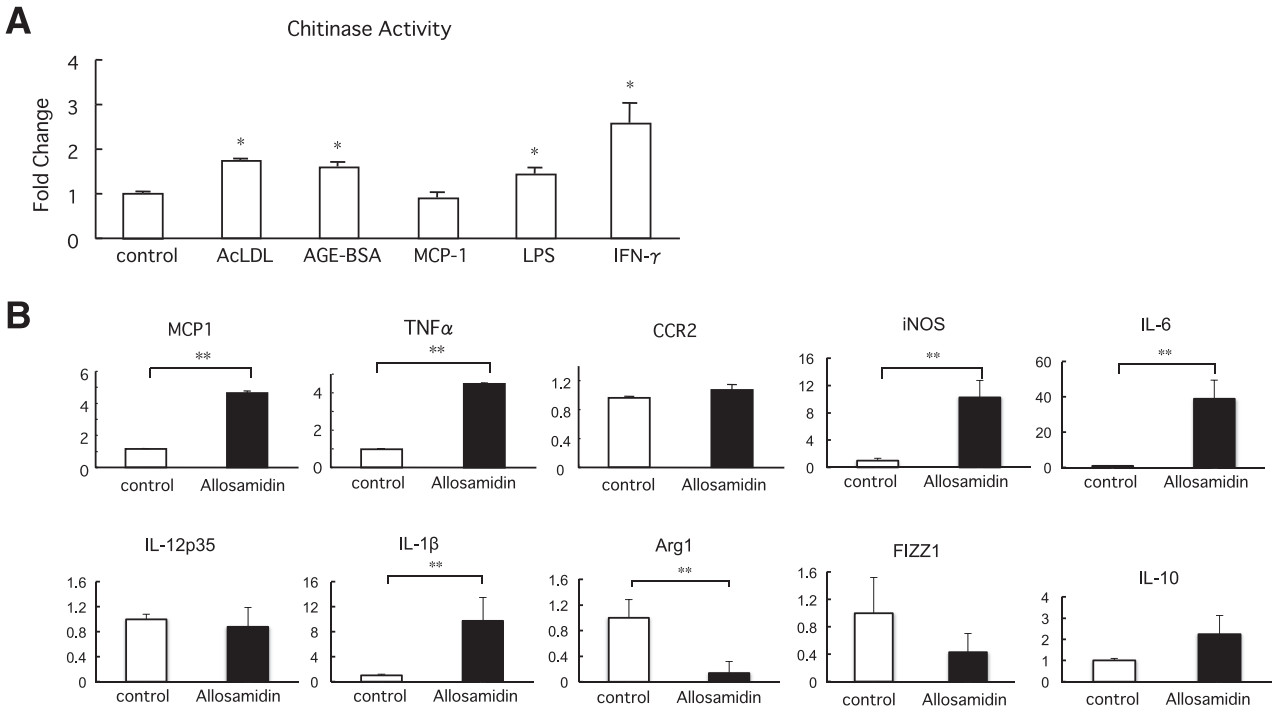

Figure 1 **A:** Chitinase activity increased in BMDM treated with Ac-LDL, AGE-BSA, LPS, and IFN-γ. BMDM from C57BL/6 mice were isolated and stimulated with 50 μg/mL AcLDL, 100 ng/mL MCP-1, 100 ng/mL LPS, 100 μg/mL AGE-BSA, or 100 units/mL IFN-γ. After 24 hours of stimulation, chitotriosidase activity in the media was measured as described in *Materials and Methods*. **B:** Chitinase inhibitor modifies expression of pro-inflammatory cytokines and polarizes macrophages into M1 phenotype. RAW264.7 macrophages were treated with medium alone or 10 μmol/L allosamidin for 24 hours before RNA isolation. Transcripts for TNFα, MCP-1, CCR2, inducible nitric oxide synthase, IL-6, IL-12p35, IL-1β, Arg1, FIZZ1, and IL-10 were quantified by real-time PCR. Results are representative of at least three independent experiments and values are expressed as fold change in abundance (means ± SEM). $*P < 0.05$, $**P < 0.01$ versus control.

(PPARγ) and liver X receptor α (LXRα) expressions were also suppressed by allosamidin treatment (Figure 3A). In accordance with these results, allosamidin suppressed receptor-specific binding and uptake of acLDL (Figure 3B). Moreover, cholesterol efflux assay showed that ApoAI-specific cholesterol efflux was significantly decreased by allosamidin treatment (Figure 3C). HDL-specific cholesterol efflux tended to decrease, although not significantly, and the increase in percentage of HDL-specific efflux was significantly reduced by allosamidin treatment (Figure 3C).

The Effects of Specific CHIT1 Inhibition and CHIT1 Overexpression Resemble that of Allosamidin in Primary Macrophages

To elucidate whether the allosamidin treatment would have similar effects in primary macrophages as in RAW264.7 cells, we treated BMDM with allosamidin ± IFN-γ stimulation. To inhibit CHIT1 mRNA expression specifically, we also transfected thioglycollate-elicited peritoneal macrophages with siRNA against CHIT1. We used peritoneal macrophages in this experiment because the high CHIT1 expression in BMDM made it difficult to be inhibited by siRNA transfection. CHIT1 mRNA expression was decreased by approximately 60% after CHIT1 siRNA transfection (Supplemental Figure S3A). We also transfected BMDM with pcDNA3.1-CHIT1 plasmid to investigate whether CHIT1 overexpression would have the opposite effect to allosamidin treatment or CHIT1 siRNA transfection. Chitinase activity was increased by approximately 40% after CHIT1 plasmid transfection (Supplemental Figure S3B). With cytokine/chemokine gene expression, allosamidin treatment had similar effects in BMDM as in RAW264.7 cells, especially when stimulated with IFN-γ (Figure 4). Most of the genes related to cholesterol metabolism were also significantly reduced by allosamidin in BMDM (Figure 5). CHIT1 siRNA transfection tended to resemble the effects of allosamidin in cytokine/chemokine gene expression (Figure 4) and significantly reduced ABCA1, ABCG1, PPARγ, and LXRα expression, but CD36 and SR-AI mRNA expression were not changed (Figure 5). Although CHIT1 transfection experiments yielded mostly opposite cytokine/chemokine gene expression results compared to allosamidin treatment (Figure 4), only ABCA1 mRNA expression was significantly increased among the genes related to cholesterol metabolism.

Allosamidin Treatment Promotes Atherosclerosis in ApoE-Deficient Hyperlipidemic Mice

When we evaluated CHIT1 mRNA expression in the atherosclerotic aortas of ApoE knockout mice, CHIT1 mRNA expression tended to decrease in the mice fed a high-fat diet with angiotensin II infusion compared to the

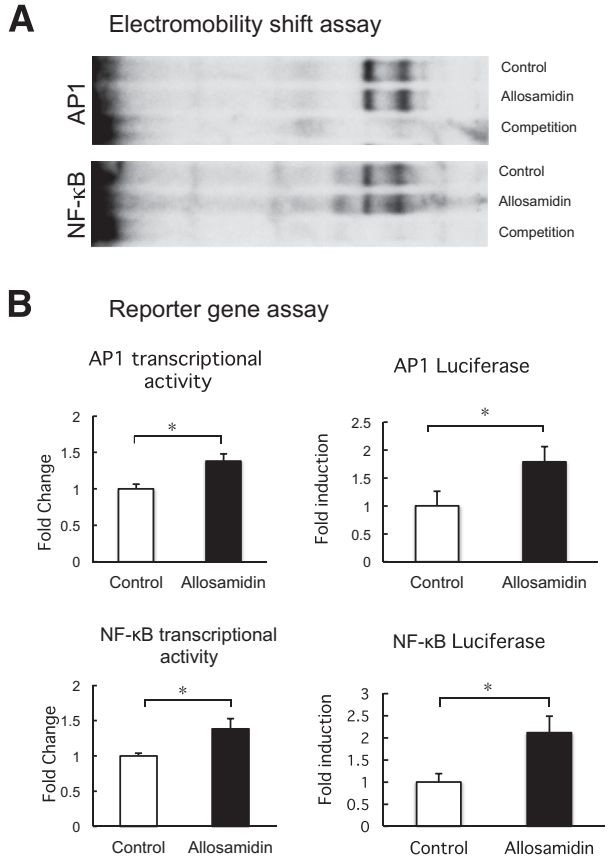

Figure 2 Chitinase inhibitor increases AP-1 and NF-κB transcriptional activity. **A**: RAW264.7 macrophages were treated with medium alone or 10 μmol/L allosamidin overnight. Nuclear protein was extracted and transcriptional activity of AP-1 and NF-κB were accessed by electromobility shift assay. **B**: RAW264.7 macrophages were transfected with AP-1 or NF-κB promoter—luciferase plasmid and 2 μg of β-galactosidase gene driven by SV40 promoter-enhancer sequence. The cells were then treated with medium alone or 10 μmol/L allosamidin overnight, and luciferase activity and β-galactosidase were measured. $*P < 0.05$ versus control.

mice fed a chow diet (Figure 6A). To investigate the role of CHIT1 in the pathogenesis of atherosclerosis, we spontaneously treated hyperlipidemic ApoE-deficient mice with allosamidin, while feeding them the Western diet for 6 weeks. Because allosamidin has been reported to inhibit chitinase activity at doses as low as 1 mg/kg *in vivo*,[14] we treated the mice with 1 mg/kg per day using the Alzet osmotic mini pump model 2006 (ALZET). Plasma chitinase activity was suppressed by approximately 40% during the experiment (data not shown) and just before euthanasia (Figure 6B). There were no significant differences in body weight change, blood pressure, heart rate, and plasma lipid profile between the control and allosamidin-treated group (Table 2 and Supplemental Figure S4). Administration of allosamidin aggravated atherosclerotic lesion area in the aortic arch (Figure 6C). There was very little plaque formation in the rest of the aortas for both groups. The atherosclerotic lesion area and macrophage infiltration area in the aortic sinus were also increased by allosamidin treatment (Figure 6D). There were very few infiltrated T lymphocytes in the plaque with no statistical difference between the two groups (Figure 6D).

Discussion

Several studies, including ours, have found that chitinase activity is increased in the sera and plaque tissue of atherosclerosis patients and nonhuman primates.[4,5,15] However, it has not been demonstrated whether there is a correlation between chitinase activity and the pathogenesis of atherosclerosis. Because CHIT1 is abundantly produced by activated macrophages, and because macrophages play an important role in the pathogenesis of atherosclerosis,[1,16] we sought to determine the effects of CHIT1 on macrophage function related to atherosclerosis. We used allosamidin to inhibit chitinase activity and investigated whether allosamidin modulates production of pro-inflammatory cytokines and cholesterol metabolism in macrophages using both cultured cells and an *in vivo* atherosclerosis model. We also inhibited CHIT1 gene expression by CHIT1 siRNA transfection and overexpressed CHIT1 protein by transfection of a CHIT1 expression vector to evaluate the specific role of CHIT1. Our data suggest that CHIT1 regulates atherosclerotic development by mediating: i) pro-inflammatory cytokine productions, ii) macrophage polarization, and iii) cholesterol uptake and efflux in macrophages.

Chitinase activity is reported to be elevated in atherosclerosis patients and is correlated to atherothrombotic stroke and ischemic heart disease.[4,5,15,17,18] Serum chitinase activity is also increased in several human lysosomal lipid storage diseases including Gaucher disease and Niemann-Pick disease. It has been proposed as an indicator of disease severity acting as a biochemical marker of macrophage accumulation and activation.[19–22] In all of these diseases, including atherosclerosis, the common theme is abnormal lipid-laden macrophage accumulation in tissues and robust secretion of CHIT1 protein.[19] CHIT1 mRNA expression is further increased by stimulation with TNFα, IFN-γ, and LPS.[13,23] Oxidized low density lipoprotein also upregulates chitinase-3-like protein 1 (gp-39) and CHIT1 in cultured macrophages whereas nonmodified LDL does not.[24] Our results show that Ac-LDL, as well as IFN-γ and LPS, increased chitinase activity in BMDM, implicating that lipid accumulation and pro-inflammatory cytokine stimulation may induce upregulation of CHIT1 expression in atherosclerotic lesions. Additionally, AGE-BSA also increased chitinase activity in the current study, indicating that the hyperglycemic state, such as diabetes mellitus, may increase CHIT1 expression as well.

There are two major types of macrophage activation: one that promotes inflammation (referred to as classically activated, or M1), and the other that promotes resolution (referred to as alternatively activated, or M2).[25] An imbalance of their activation status can influence lipid metabolism,

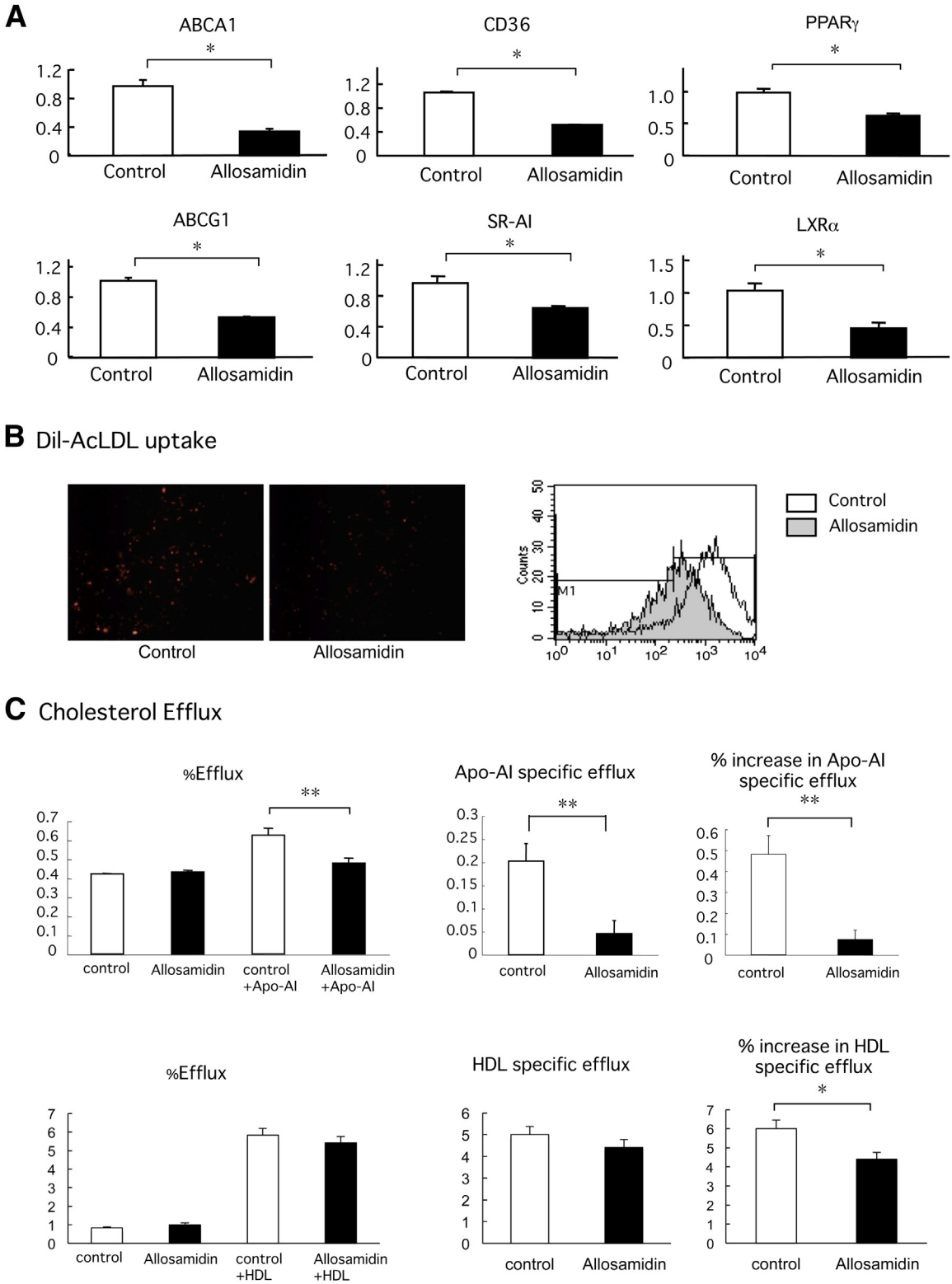

Figure 3 Chitinase inhibitor modifies expression of genes related to cholesterol metabolism and decreases cholesteryl ester uptake and cholesterol efflux in macrophage. **A**: RAW264.7 macrophages were treated with medium alone or 10 μmol/L allosamidin for 24 hours before RNA isolation. Transcripts for ABCA1, ABCG1, CD36, SR-AI, PPARγ, and LXRα were quantified by real-time PCR. $^*P < 0.05$ versus control. **B**: Chitinase inhibitor lowers cholesteryl ester uptake by RAW264.7 cells. Cells were treated with or without allosamidin overnight followed by incubation of Dil-AcLDL at 10 μg/mL in medium for 2 hours at 37°C. Intensity of fluorescence was determined by either confocal microscopy or flow cytometry. **C**: Chitinase inhibitor decreases apoAI and HDL-mediated cholesterol transport. RAW264.7 cells were labeled with ^{3}H-cholesterol for 36 hours in the presence or absence of 10 μmol/L allosamidin. ApoAI- or HDL-dependent or independent cholesterol efflux was measured by incubating [^{3}H] cholesterol-labeled RAW264.7 cells with or without apoAI or HDL. Radioactivity in the media was determined as a percentage of total radioactivity in the cells and media ($n = 3$; means ± SEM). $^*P < 0.05$, $^{**}P < 0.01$ versus control.

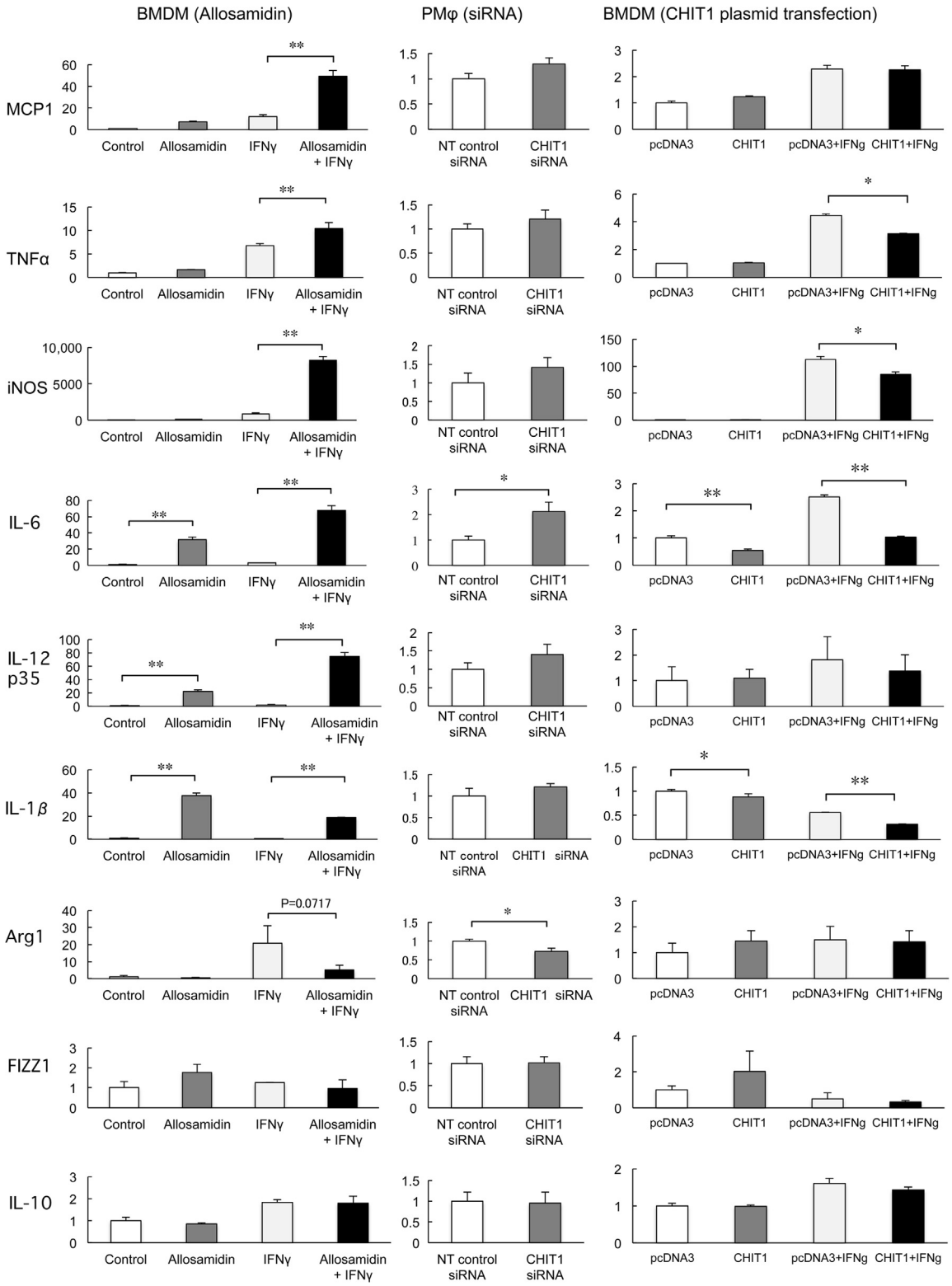

Figure 4 Chitinase inhibition, CHIT1 inhibition, and CHIT1 overexpression modify pro-inflammatory cytokines and polarize primary macrophages toward a M1 phenotype. BMDM were treated with medium alone or 10 μmol/L allosamidin for 24 hours and were stimulated with or without 10 units/mL IFN-γ for 6 hours before RNA isolation. Thioglycollate-elicited peritoneal macrophages were transfected with siRNA against mouse CHIT1 or nontargeting siRNA using lipofectamine siRNA maximum. After 48 hours of transfection, the cells were harvested for mRNA isolation. BMDM were also transfected with control pcDNA3.1 or pcDNA3.1-CHIT1 plasmid by electroporation (MicroPorator MP-100). After 48 hours of transfection, the cells were stimulated with or without 10 units/mL IFN-γ for 6 hours and harvested for mRNA isolation. Transcripts for TNFα, MCP-1, inducible nitric oxide synthase, IL-6, IL-12p35, IL-1β, Arg1, FIZZ1, and IL-10 were quantified by real-time PCR. The results are representative of at least three independent experiments and values are expressed as fold change in abundance (means ± SEM). PMφ, thioglycollate-elicited peritoneal macrophages. $*P < 0.05$, $**P < 0.01$ versus control.

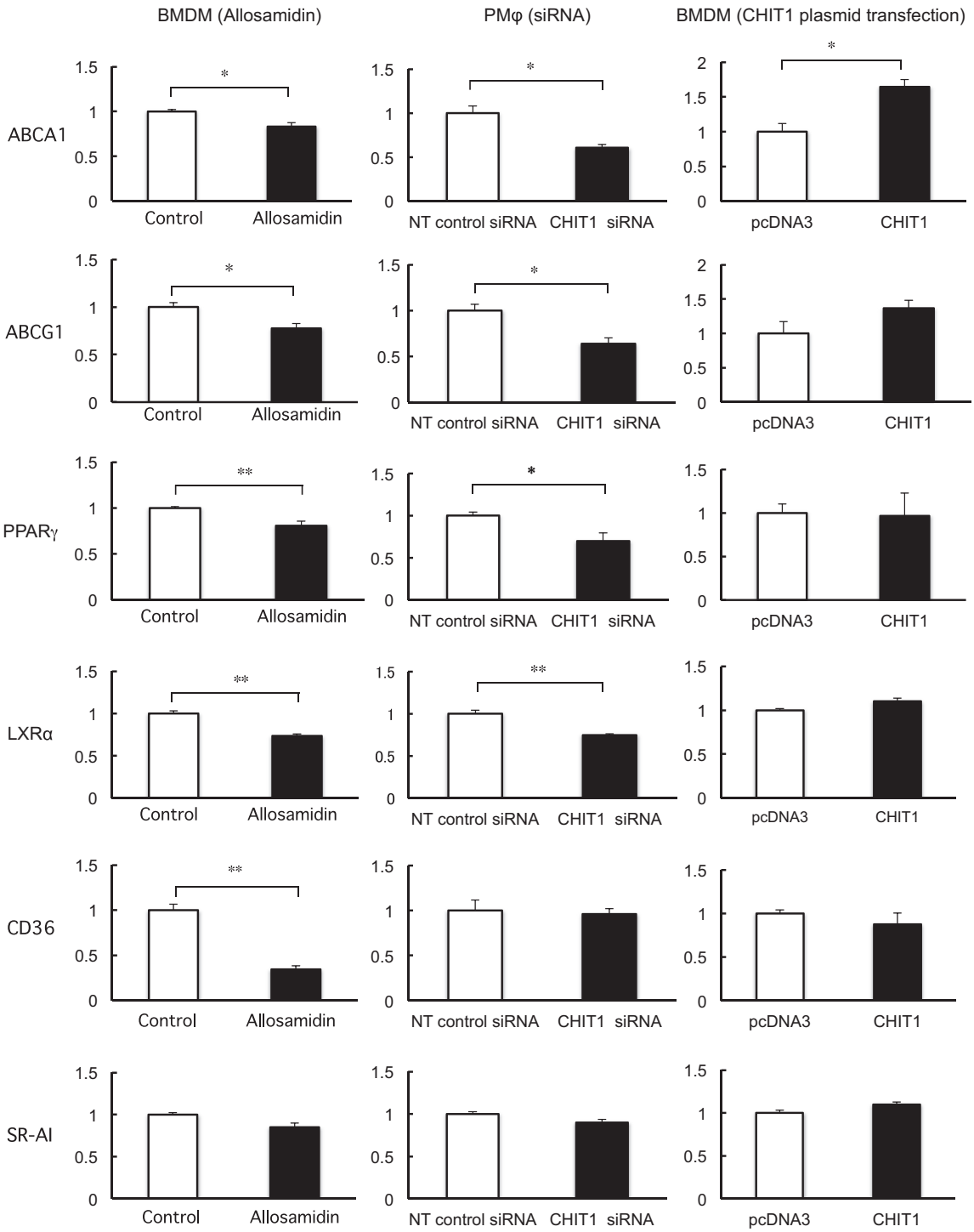

Figure 5 Chitinase inhibition, CHIT1 inhibition, and CHIT1 overexpression modify expression of genes related to cholesterol metabolism in primary macrophages. Transcripts for ABCA1, ABCG1, CD36, SR-AI, PPARγ, and LXRα were quantified by real-time PCR. The results are representative of at least three independent experiments and values are expressed as fold change in abundance (means ± SEM). PMφ, thioglycollate-elicited peritoneal macrophages. $*P < 0.05$, $**P < 0.01$ versus control.

inflammatory responses, and plaque stability, and thus can affect the progression and complication of atherosclerosis.[26] Because T helper-2 cytokines, such as IL-10, can shift the balance in favor of M2 macrophages and have beneficial effects on atherosclerosis, M2 polarization is thought to be atheroprotective.[11,25,27–29] Gaucher cells, which display elevated CHIT1 expression in response to lipid accumulation, resemble anti-inflammatory M2 macrophages.[30] Interestingly, another member of the chitinase family, the acidic mammalian chitinase, is reported to mediate T helper-2-specific, IL-13-induced asthma.[14] Taken together, these findings implicate CHIT1 as a mediator of M2 macrophage

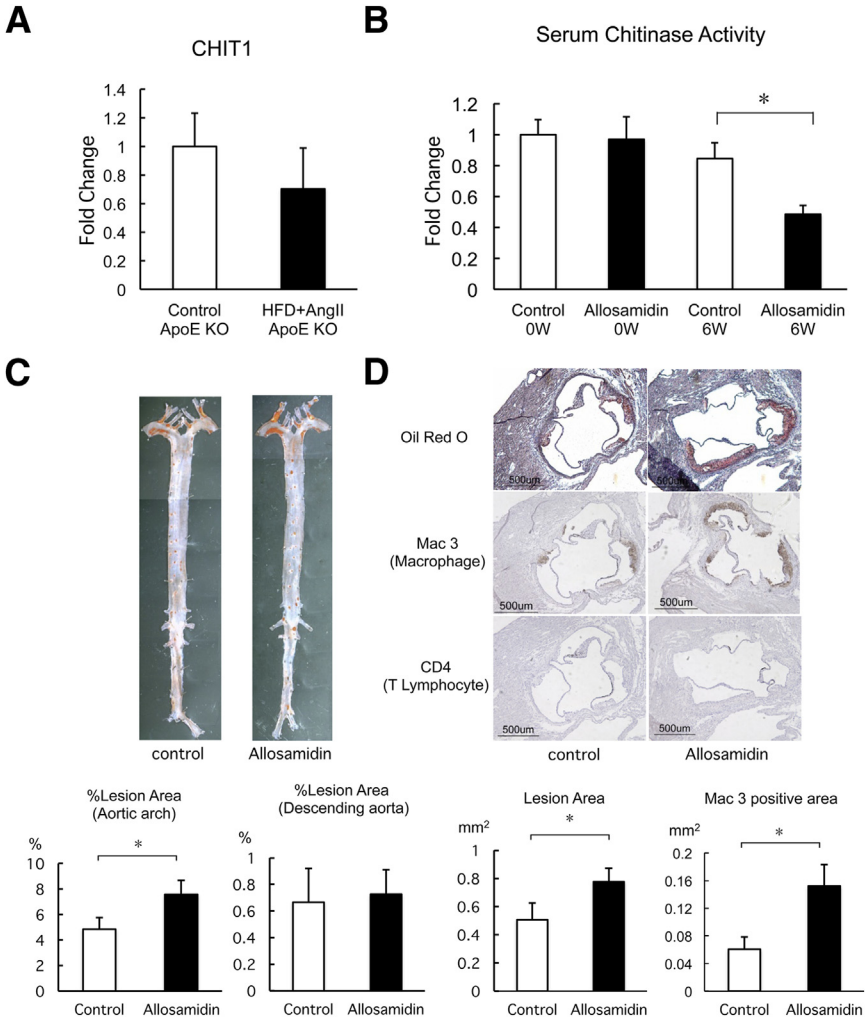

Figure 6 Chitinase inhibitor reduces atherosclerotic lesions in spontaneous ApoE-deficient hyperlipidemic mice fed a Western diet. **A:** CHIT1 mRNA expression in atherosclerotic aortas of ApoE-deficient mice fed a chow diet or high-fat diet with 1.9 mg/kg per day infusion of angiotensin II for 4 weeks. The descending aortas were harvested and total mRNA was extracted. CHIT1 mRNA expression was evaluated by real-time RT-qPCR ($n = 7$ in control group; $n = 8$ in high-fat diet + angiotensin II group; means ± SEM). **B:** The 12-week-old mice were implanted with an osmotic mini pump model 2006 (ALZET) containing 10 mg/kg per day allosamidin or control vehicle, and fed a Western diet for 6 weeks. Serum chitinase activity was measured before and after 6 weeks of allosamidin treatment. Allosamidin administration suppressed serum chitinase activity by approximately 40%. **C:** Aortas (aortic arch to the iliac bifurcation) from control and allosamidin-treated mice fed the atherogenic diet for 6 weeks were opened and stained *en face* with Oil Red O (Sigma-Aldrich). Representative images of the aortas showing the surface plaque (top) and quantification of surface area occupied by lesions in the aortic arch (bottom left) and the descending aortas (bottom right) are shown. Data (percentage of area occupied by lesions) are expressed as means ± SEM for each group. **D:** Aortic sinus atherosclerosis in control and allosamidin-treated mice. Atherosclerotic lesion in the aortic sinus from cryosections was stained with Oil Red O and counterstained with H&E. Sections were also stained with Mac-3 or CD4 antibody coupled with horseradish peroxidase-labeled secondary antibody and counterstained with hematoxylin to identify the macrophages and T lymphocytes present in the aortic sinus lesions. Representative images for each staining are shown. The atherosclerotic lesion area stained with Oil Red O and the macrophage infiltration area stained with Mac-3 antibody were quantitated using a computer-assisted imaging system. $^*P < 0.05$.

phenotype and T helper-2 response. In agreement with this, our data show that allosamidin treatment increased the expression of M1 markers and decreased the classic M2 marker, suggesting that CHIT1 polarizes macrophages into the anti-atherosclerotic M2 phenotype.

Cholesterol homeostasis within macrophages is determined by the balance between the uptake of modified lipids via the scavenger receptors SR-A and CD36, and cholesterol efflux via the reverse cholesterol transporters ABCA1 and ABCG1. Overexpression of human SR-A in bone marrow cells attenuated atherosclerosis in low density lipoprotein receptor-deficient or ApoE-deficient mice,[31,32] whereas SR-A deficiency in atherogenic apoE3-Leiden mice led to more severe and complex atherosclerotic lesions.[33] Loss of lipid uptake via SR-A or CD36 may lead to the formation of necrotic core within atherosclerotic lesions.[34] These reports suggest that lipid uptake via scavenger receptors may be protective against atherosclerosis. ABCA1 and ABCG1 mediate the critical part of cholesterol efflux from lipid-laden macrophages to HDL cholesterol.[35,36] Patients with Tangier disease (due to loss of functional mutations in the ABCA1 gene) show low serum HDL cholesterol level, and have a moderately increased risk for coronary artery diseases.[37,38] Similarly, deficiency of either ABCA1 or ABCG1 in atherogenic hyperlipidemic mice led to massive lipid accumulation in macrophages and increased atherosclerotic lesions,[39,40] suggesting that cholesterol efflux via ABC transporters is anti-atherogenic. In our study, allosamidin treatment led to decreased SR-AI,

Table 2 Various Measurements in Mice Treated with Allosamidin versus Control Mice

	Control	Allosamidin
n	6	6
Body weight, g	36.9 ± 1.2	35.8 ± 2.4
BP, mmHg	115/67 ± 4.8/8.3	115/71 ± 5.2/9.9
HR, bpm	588 ± 47	597 ± 44
Plasma total cholesterol, mg/dL	908.6 ± 76.8	929.8 ± 114.4
Plasma total triglycerides, mg/dL	134.0 ± 34.1	140.4 ± 38.1

No measurements were statistically significant.

CD36, ABCA1, and ABCG1 expression, which presumably resulted in suppressed cholesterol uptake and efflux from macrophages, leading to exacerbation of atherosclerosis.

Although a detailed molecular mechanism of the effects of CHIT1 is not fully elucidated in this study, its effects on immune response and cholesterol metabolism appear to involve the PPARγ-LXRα pathway. PPARγ has anti-inflammatory properties via negative interference with NF-κB, AP-1, and signal transducer and activator of transcription.[41] Furthermore, positive correlation was observed between PPARγ expression and the M2 markers, such as CD206, CD36, and CC-chemokine 1.[42–44] Additionally, activation of PPARγ and/or LXRα upregulates ABCA1 and ABCG1 expression,[35,45] and PPARγ agonists curb atherosclerosis by inhibiting foam cell formation.[46] Because our data show that expression of PPARγ and LXRα is decreased by allosamidin treatment, it is likely that CHIT1 exerts its anti-inflammatory effects and promotes cholesterol efflux from macrophages via activation of the PPARγ-LXRα pathway. The detailed mechanism of PPARγ and LXRα upregulation by CHIT1 is currently being investigated.

Whereas serum CHIT1 activity is reported to increase in patients with atherosclerosis and is correlated with the severity of the disease,[4,5,15] at least in the Corsica Island case-control study of patients with coronary artery disease and healthy subjects showed that the frequency of loss of function mutation in *CHIT1* gene is not correlated with the disease.[47] Similarly, because the range of serum CHIT1 activity values from control subjects is wide and in many cases overlaps with those from atherosclerosis patients,[5] it seems that these data do not necessarily indicate that *CHIT1* is just a marker of atherosclerosis. Further clinical studies are required to elucidate the physiological role of CHIT1 in human atherosclerotic diseases.

There are several limitations in interpreting our study to speculate CHIT1 functions in atherosclerosis. First, allosamidin is a pseudotrisaccharide consisting of two $\beta^{1,4}$-linked *N*-acetyl allosamine sugars coupled to allosamizoline, which renders it an effective inhibitor of all members of the chitinase family 18.[6,48] Thus, another human chitinase, acidic mammalian chitinase, as well as family 18 chitinase-like mammalian proteins without chitinase activity, such as YM1 and gp-39, were likely inhibited by allosamidin in our *in vivo* study. Because expression of gp-39 is also reported to be strongly upregulated in macrophages in atherosclerotic lesions,[4] it is possible that allosamidin aggravated atherosclerotic lesions partly by inhibiting gp-39 function. Also, due to limitations in allosamidin delivery, atherosclerosis examined in the current study was not very advanced.

In summary, continuous administration of a chitinase inhibitor, allosamidin, for 6 weeks increased atherosclerotic lesions in hyperlipidemic mice. Allosamidin treatment caused increased expression of pro-inflammatory cytokines, including TNFα and MCP-1, induced M1 macrophage polarization, and decreased lipid uptake and cholesterol efflux in macrophages via down-regulation of SR-AI, CD36, ABCA1, and ABCG1 expression. These data suggest that CHIT1 has a protective role against atherosclerosis, and that measures taken to enhance the local chitinase activity in atherosclerotic lesions may provide a novel treatment for atherosclerosis.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.04.003*.

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